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Risk factors for the development of breast cancer remain largely unknown, especially those associated with the effect of the environment. Despite the known influence of hormonal factors, breast tumorigenesis has been shown to involve an accumulation of serial genetic changes in cancer genes. X-irradiation is a genotoxic exposure that has been identified as a risk factor for breast cancer. If somatic mutation is a major mechanism that drives the process of breast carcinogenesis, we hypothesize that breast cancer should develop preferentially in women with higher frequencies of somatic variation, as measured at a neutral reporter locus. The rate of somatic mutation is determined by exposure to endogenous and exogenous genotoxic agents and by an individual's ability to deal with such exposures through biotransformation of xenobiotics and DNA repair. We are using two established bioassays of *in vivo* human somatic mutation to study a large population of newly diagnosed primary breast cancer patients. These genetic/environmental interactions can be quantitated simply from observed effect, before we have completely understood the underlying mechanisms. Our primary goal is to provide a rationale for simple laboratory blood tests to be incorporated into the risk estimation equation for breast cancer.

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Introduction

Breast cancer is one of the most common malignancies in the western world, and increases in incidence are being seen worldwide. Risk factors for the development of breast cancer remain largely unknown, with up to 75% of breast cancers occurring in the absence of established risk factors. The three risk factors that have been established are: family history of breast cancer, metabolic factors related to hormone production, and exposure to X-irradiation (Gail and Benichou, 1992; Claus et al., 1994). This study proposes that the last mentioned factor, exposure to ionizing radiation, is actually indicative of a larger involvement of genotoxicity in the etiology of breast cancer. This suggests that exposure to carcinogenic chemicals and susceptibility to mutagenesis and carcinogenesis play a significant role in determining who will develop a tumor of the breast. Indeed, evidence is mounting that the two known breast cancer susceptibility genes, BRCA1 and BRCA2 are actually DNA repair genes that modulate the genotoxic effects of environmental exposures (Scully et al., 1997; Sharan et al., 1997). Although there is bound to be some element of tissue specificity for both genotoxic exposure and susceptibility to DNA damage, it is impractical to monitor genotoxic exposure in breast tissue itself. Blood, however, and its progenitor tissue bone marrow, are present throughout the body, and most xenobiotic exposures to the breast are likely to be transported to the breast tissue through the blood. We have therefore proposed to assay for somatic mutation, the ultimate outcome of genotoxic DNA damage, in blood samples from newly diagnosed breast cancer patients and matched controls. We are applying two such assays, mutation at the HPRT gene in lymphocytes and at the GPA gene in erythroid cells. These are the only two widely applied (and applicable) assays for human somatic mutation (Grant and Jensen, 1993). The association between human carcinogenesis in general, and somatic mutation as measured with the *HPRT* assay has recently been discussed by Simpson (1997), and hepatocellular carcinoma patients have been shown to exhibit elevated somatic mutation frequencies with the *GPA* assay (Okada et al., 1997). These studies are similar to those of Spitz and co-workers, who have shown that sensitivity to bleomycin (a crosslinking agent used to diagnose the cancer-prone syndrome Fanconi anemia) (Wu et al., 1996), and benzo[a]pyrene diol epoxide (a mutagenic component of tobacco smoke) (Wei et al., 1996) are risk factors for cancers of the lung and of the head and neck. The mutagenicity assays proposed in the present study are much less specific to particular environmental agents than those of Spitz, and are therefore more appropriate for application to breast cancer, where there is much less information on potential causative genotoxic exposures.

Body

In the present study, we will establish that there is indeed a correlation between measurable somatic mutation frequencies at these reporter genes in the blood and breast cancer incidence, presumably as a result of concurrent accumulation of mutation at breast cancer-related oncogenes (Bieche and Lidereau, 1995). We propose to apply both mutation assays to the same population to determine the strength of the correlation between mutation at these two loci in a population lacking a known genotoxic exposure. In studies of known exposures to both radiation and chemicals, the correlation of the *GPA* and *HPRT* mutant frequencies is very high, indicating that individual factors modifying the mutational response affect both biomarkers similarly (Perera et al., 1993; Hirota et al., 1994). This study, unlike those compiled in Grant (2001), is designed specifically to compare newly diagnosed breast cancer patients to age-matched controls. Therefore, we can determine whether somatic mutational burden might serve as a significant risk factor for breast cancer incidence, when taken in the context of the known breast cancer risk factors currently applied through the Gail and Claus breast cancer risk models (Gail and Benichou,

1992; Claus et al., 1994). The data published in Grant (2001) is that originally presented as preliminary studies for this grant. It consists of a meta-analysis of HPRT data from the literature, as well as our own GPA data from several studies of heterogenous cancer patients that were to be treated with known genotoxic chemotherapies.

The original submission of this grant was done under the auspices of MWH 93-108, an IRB that allowed for blood sampling and administration of a questionnaire detailing exposure history and known risk factors for patients with breast, colon, endometrial and ovarian cancer. This protocol allowed for a number of different assays of "genomic stability", including the HPRT and GPA somatic mutation assays, assays of DNA repair capacity, measurements of mutagen sensitivity, etc. by a group of collaborators. Much of the past year has involved the development of an IRB protocol specifically designed for this study, with myself as the principal investigator, with the same name as this grant and with no provisions for further studies not detailed in this grant. This was required by new regulations governing IRB protocols. Such an IRB has been submitted for expedited review and will go into effect for August, 2001 (perhaps it was in anticipation of such problems that the study section insisted that the study would take longer than I had initially proposed). This experience prompted me to develop and submit a DOD Core grant proposal ("Mitogenesis, Mutagenesis and their Interaction in the Etiology of Breast Cancer") that would fund a research infrastructure for our Comprehensive Cancer Center, administering questionnaires to all consenting breast cancer patients and providing a bank of blood and viable cell samples for investigators working on the interaction between hormonal and genotoxic risk factors in the development of breast cancer. The faculty of this Core are required to share data on their multiple endpoints in order to better detect interactions that might be crucial to the development of breast cancer, and that might allow for optimal intervention and prevention.

Given that we could not sample patients until our new IRB was accepted, we recruited and trained our staff. Ms. Michelle Huerbin (B.S., M.P.H. in Health Services Administration) was hired as Research Coordinator. She previously performed this role for the University of Pittsburgh Cancer Institute/Magee-Womens Hospital Cancer Genetics Program, where she coordinated studies of breast cancer genetics (specifically BRCA1 and BRCA2 heterozygosity) and risk assessment. Ms. Huerbin also has experience as a laboratory technician, and has been trained in the performance of the flow cytometric GPA assay. Ms. Britt Luccy, the technician on the original proposal, has gone on to graduate school at Johns Hopkins, her replacement is Ms. Julie Conte, who was an intern in my laboratory last summer, and is proficient in the HPRT assay.

HPRT training has been performed on established lymphoblast cell lines, and, as detailed in the M.S. thesis of my student Mr. Bob Babra, our laboratory experience pointed towards an important correlation between cell viability (expressed as "cloning efficiency", CE) and mutation frequency. In order to see whether CE was also an important factor in population studies, we analyzed HPRT mutation frequency data from a large cohort of smokers and "matched" non-smokers published by Jones et al. (1993), the largest such study that provided individual CE for each subject. The results, given in Table 1, demonstrate that while aging and smoking status are usually considered characteristics that should be matched in epidemiological studies of mutation, cell viability actually has a far greater effect. This effect was reported in Cole et al. (1988), who advised that any assay with a CE of less than 30% should be considered invalid. The reason this advice is ignored is probably practicality. Unlike smoking status or age, CE cannot be determined prior to the performance of the assay, so that Cole et al. are actually advising investigators to invalidate assays that may represent half of their data (if the Jones et al. data in

Table 1 are representative). Instead, we suggest that patients should be matched to controls of similar CE. Since this cannot be done a priori, it requires the development of a database of controls with a distribution of ages, smoking histories and cell viabilities.

Table 1. Potential factors affecting HPRT somatic mutation frequency

Population	N	HPRT M_f ($\times 10^{-6}$)		Range	
		Mean	Median		
Jones et al. normal adults	120	8.5 ± 5.9		6.9—36.5	
Jones et al. normal adults (CE > 30%)	60	6.7 ± 4.6		5.8—28.4	
Jones et al. normal adults (CE < 30%)	60	10.3 ± 6.5	7.5	2.1—36.5	$p < 0.0001$
Jones et al. normal adults (nonsmokers)	62	7.5 ± 5.9		5.7—36.5	
Jones et al. normal adults (smokers) 58		9.6 ± 5.3		7.5—24.1	$p = 0.055$
Jones et al. (nonsmokers, CE > 30%)	30	6.2 ± 4.9		5.0—28.4	
Jones et al. (nonsmokers, CE < 30%)	32	8.7 ± 7.2		6.7—36.5	$p = 0.11$
Jones et al. (smokers, CE > 30%)	30	7.2 ± 4.3		6.8—24.1	
Jones et al. (smokers, CE < 30%)	28	12.1 ± 5.0	12.2	4.4—21.3	$p = 0.0002$

Key Research Accomplishments

Key accomplishments for this first year include the recruitment and training of study personnel. The development of an IRB protocol specific to this project that satisfies the funding agency and the local IRB committee and is considered feasible by our study personnel and our clinical colleagues. Publication of the retrospective data that supported the funding of the grant. The finding of an important confounding effect, CE, in our training data that will improve our ability to demonstrate subtle effects in the greater study.

Reportable Outcomes

Successful funding of this project allowed me to publish our preliminary results in Grant (2001). Training data from personnel on this study contributed to the M.S. thesis of Bob Babra in the department of Environmental and Occupational Health at the University of Pittsburgh ("Somatic Mutation and Cancer: Induction, Predisposition and Therapy"). A manuscript is in preparation.

Conclusions

Problems with the start-up of this project have been addressed by the design of a new, independent IRB. Similar problems have potentially been addressed in the long term with the submission of a DOD Core research infrastructure grant. The clinical breast cancer population at Magee-Womens Hospital continues to increase with the further consolidation of breast care for the 20-hospital University of Pittsburgh Health System HMO at Magee. We have greater facility with our assays and a team in place to process samples and data. In addition, my laboratory is now located directly across the street from Magee-Womens Hospital (as opposed to 11 miles away), which should further facilitate access to valuable patients and samples. The acquisition of samples will now follow the original three-year timetable that requires successful processing of approximately two samples per week for the duration of the grant.

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Appendices

Grant SG (2001) Molecular epidemiology of human cancer: biomarkers of genotoxic exposure and susceptibility. *Journal of Environmental Pathology, Toxicology and Oncology* (in press).

Molecular Epidemiology of Human Cancer: Biomarkers of Genotoxic Exposure and
Susceptibility

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Abstract

The new field of “molecular epidemiology” attempts to investigate the link between toxic exposure and an associated health effect by defining presumptive intermediate stages in the development of the disease state based on known mechanisms. In the development of malignancy, these steps may involve: exposure to known mutagens and carcinogens, internalization and potentially metabolism of a chemical agent, characterization of the interaction of the agent at its site of action (usually DNA), characterization of induced preneoplastic changes and, in certain instances, early detection of the cancer itself. These processes can be monitored through biomarkers specific to each of the steps in the progression towards disease, and a host of applicable techniques are now available. An overview of such techniques is presented, with an emphasis on techniques offering insight into the malignant process itself. Evidence is presented that suggests that although there are many potential contributing mechanisms to carcinogenesis, mutagenesis remains the dominant driving force behind the process. Several methods of monitoring mutation have shown promise as predictors of cancer incidence. These methods might also be used as monitors of agents designed to intervene in the process and prevent the development of overt disease.

Running Title: Biomarkers of human environmental carcinogenesis

Key Words: Genetic toxicology, carcinogenesis, mutagenesis, GPA, HPRT, DNA repair, mutagen sensitivity

Introduction

It has become fashionable to place the word “molecular” before the name of a classical field of scientific research and consider it reinvented. This, often in the absence of what the word actually means in this context, and how this refined and redefined field truly differs from its progenitor. In the case of “molecular” toxicology, there has been a real shift from the traditional activity of testing chemical toxicity in model systems to studies in the true organism of interest, man. These human studies have their own advantages and disadvantages; they are, by definition, epidemiological, and epidemiology is very different from experimental science, requiring larger, more expensive and more interdisciplinary studies. Often the investigator has no control over the agent of exposure or the dose or doses administered; indeed, in most cases, one must rely on “found” experiments, such as accidental exposures, which is often uncomfortably similar to ambulance chasing. For the accumulation of significant data, more than an anecdotal case report is required, so there must be a relatively large exposed population, with a considerable increase in incidence of disease. Indeed, this field often relies upon the pharmaceutical industry to provide a large population of exposed individuals exhibiting unanticipated toxic effects. This relative inability to study an agent of the investigator’s choice is offset by the fact that real human beings are the source of data, so that there is no question as to the applicability of the “model” system. These studies therefore involve what has become known in biomedical science as “translational” research, i.e. science that has direct application in real-life situations.

In the context of public health, the promise of molecular toxicology and molecular epidemiology is the possible identification of an impending disease state prior to clinical manifestation, potentially allowing for biological, chemical or behavioral intervention and, perhaps, prevention. This is a particularly appropriate approach to cancer, since many avenues of research have shown carcinogenesis to be a multi-step process with a duration or gestation time of decades. Cancer can occur due to the delayed effects of a single short-term exposure, such as a radiation accident, or due to the effects of an otherwise asymptomatic chronic exposure. In this delayed or accumulative aspect of its etiology, it is very possible that cancer can act as a paradigm for other late-onset diseases, in that somatic effects are more important factors in the development of the disease than genetic predisposition. With the impending completion of the human genome

project, however, attention has increasingly moved towards these genetic factors, even in diseases of aging. Besides the many technological tools being developed in this area, such as gene expression and polymorphism "chips", the main reason for this concentration on genetics is that it can be fully ascertained at any age. For example, a blood sample from a 80 year old contains all the genetic information that would have been available had the subject been sampled at birth. In contrast, toxicological exposures wax and wane, overlay one another and are ongoing at any point of sampling; thus, there is no easily obtainable record of overall exposure history similar to that of the underlying genetic background.

Molecular Epidemiology

In the classical toxicological epidemiology model, a defined health effect, often a well-characterized clinical disease, is perceived as occurring due to the exposure of an organism to a deleterious biological, chemical or physical agent. This strict cause-and-effect relationship is mediated through a number of unknown modifiers of exposure and response, related to the anatomy, biochemistry and physiology of the organism under consideration. The molecular epidemiological model, as delineated in **Figure 1**, attempts to expand on the concept of such biological modification by breaking the process into sequential stages that must be traversed in order to manifest disease. These intermediate stages are based on mechanistic studies and hypotheses that attempt to identify the target tissue or cell type (which may not be the same as the cell type ultimately affected by the disease, or even at the same site as the eventual disease manifestation), the response or responses necessary to convert exposure into biological effect, and, if possible, preclinical evidence of impending disease. As in any hypothetical system, it is important that there be experimentally verifiable predictions indicative of each stage. These indicators of biological modification are known as biomarkers, and since they precede clinical disease, they are thought of as "intermediate" biomarkers that can be used to monitor the progress of the disease process.

The development of the field of molecular epidemiology has been, and continues to be hindered by a lack of complete understanding and cooperation between the practitioners of the two progenitor disciplines, laboratory toxicologists and epidemiologists. For the toxicologist, the

traditional laboratory truism "if you need statistics to prove your point you didn't design the experiment properly" is difficult to reconcile with epidemiological studies. On the other hand, epidemiologists, especially clinical epidemiologists, often seem to forget that statistical associations are not and cannot be proof of causality. The proper course is for epidemiological studies to generate mechanistic hypotheses that are then evaluated experimentally. Too often there is a complete disconnection between the two disciplines. Epidemiologists hire technicians to perform tests they have seen published in the literature, often without a thorough understanding of the relevance or implications of the results. Toxicologists, on the other hand, attempt to apply their knowledge of experimental design to epidemiological studies, without an appreciation for the statistical methodologies necessary to adjust for unanticipated confounding effects. In many ways, the situation is reminiscent of the difference between academic and industrial or regulatory toxicologists: academic toxicologists apply a continually revised or "improved" protocol to a series of individual, often unrelated projects, whereas industrial and regulatory toxicologists apply a standardized, but almost always obsolete or sub-optimal protocol to a very systematic study of an area of proven concern. Thus, in collaboration, the laboratory toxicologist can address the mechanistic relevance of a biomarker to the disease of interest, troubleshoot and adapt the protocol to the types of samples that can be obtained, and offer the possibility of experimental follow-up on mechanistic hypotheses that might result from an epidemiological study. The epidemiologist, in turn, directs the study to a question of immediate concern to medicine or public health and allows for testing of both a mechanistic hypothesis and the biomarker designed to detect and monitor it in human studies.

Biomarkers of Carcinogenesis

To propose and test biomarkers of a specific disease, some insight into its etiology must be available. There have been many models of the carcinogenic process proposed: epigenetic, viral, toxicological, endocrine, immune surveillance, histopathological, etc., but the somatic mutational model (Nowell, 1976) has become predominant for a number of reasons. First, there was the discovery of dominant activated oncogenes and recessive tumour suppressor genes, and their identification in all types of cancer (Bishop, 1987; Green, 1988). Second, the linking of these mutations with histological progression, as best exemplified by the Vogelstein model of

colorectal cancer (Vogelstein et al., 1988). Third, there is the unique ability (and willingness) of the supporters of this model to integrate aspects of other models into itself. For example, the somatic mutational model has to be compatible with the viral model, since activated oncogenes were first identified in oncogenic viruses, and only subsequently were shown to have homologues in the host genome. The model is also flexible enough to allow that epigenetic changes in gene expression, such as endocrine stimulation, hyper- or hypomethylation of genes can have the same effect as mutation in fulfilling the requirements of a step in the carcinogenic pathway. Toxicologists are satisfied with the mutational model in that it describes a multistep process involving classical mutations that can be caused by radiation or electrophilic chemicals. Thus, most intermediate biomarkers of cancer presume that mutation is the only or principal mechanism of carcinogenesis and are designed to detect mutagenic exposures, premutagenic and mutagenic lesions and the biological effects of somatic mutations. Toxicologists must be reminded that not all cancer researchers are prepared to so directly equate carcinogenesis with mutagenesis, despite the fact that this principle underlies almost all "carcinogenicity" testing and costs industry billions.

Carcinogenic Exposure

Practically, there are two approaches for studying carcinogenic exposures: identification of actual exposures and identification of potential exposures. Obviously, the former is often retrospective, whereas the latter is prospective. Applied primarily to anthropogenic chemicals, a large number of carcinogenicity, mutagenicity and other types of assays have been developed to determine or predict whether a chemical is a potential human carcinogen. The gold standard is the chronic animal cancer test, with rodent carcinogenicity tests the most widely applied (Gold et al., 1997). These lifetime studies are time consuming and expensive, often have questionable application to humans, and have been increasingly criticized by animal rights activists. Attempts to establish single-cell "short-term" assays have usually been based on a mutational basis for the carcinogenic process, and measured genotoxicity (Zeiger, 1997). All of these tests suffer from fundamental oversimplifications in their basic assumptions. For example, they must assume that biological effects of exposures to multiple genotoxins (which include all *in vivo* exposures) can be estimated from additively combining the efficacy of individual constituents, suggesting

that interactions such as synergism and antagonism either do not occur, or overall, balance one another out. They also must assume that all genotoxicants have simple dose-response kinetics, ignoring the possibilities of hormesis or other higher order interactions. There are presently a huge number of man-made chemicals in use without significant toxicological data; however, there are at least two promising approaches towards improving the efficiency of testing in the near future. First is the adoption of high-throughput and/or high-content screening technologies, utilizing advances in fields such as robotics, flow cytometry, computer-directed microscopy, mass spectroscopy, etc. to better apply our current knowledge of carcinogenesis. Such technologies have already been successfully applied in some aspects of toxicology (Taylor et al., 1994; Kramer, 1998; Burchiel et al., 1999; Van Bocxlaer et al., 2000), but not to the degree they have been embraced by pharmacologists for drug design (Persidis, 1998; Silverman et al., 1998; Hopfinger and Duca, 2000; Sundberg 2000). A second promising approach towards increasing our capacity to screen chemicals has also been increasingly utilized in pharmacological drug design: the development of so-called *in silico* models, or predictive computational toxicology. Many approaches have been tried, from attempts to reproduce the logic of a working toxicologist through hierarchical sets of rules and decision trees, to correlation of chemical structure or physicochemical properties with biological activity, to artificial intelligence systems such as neural networks that attempt to combine the best features of each approach (Benfenati and Gini, 1997; Benigni and Richard, 1998; Rosenkranz et al., 1999). The challenge is much greater for toxicologists than for drug designers, however, since identification of a single successful lead compound can make the approach successful in the latter case, whereas missing a single toxic compound in the former could result in tragedy. Indeed, predictive models must continue to be developed through continuous interaction with traditional toxicologists, validating and extending models through targeted testing of new agents, and taking into account the greater considerations of the entire human organism and population (Holtzman, 2000).

The second approach to defining exposures takes place in the field, often after an exposure has occurred, or is suspected. Although this is the natural beginning of an epidemiological toxicological study, such physical measurements are traditionally the province of other disciplines, such as the industrial hygienist or the health physicist. Indeed, there is often no attempt, outside of the work environment or agents, such as radon, that are sometimes

specifically targeted by local departments of health, to measure or monitor the “normal” exposures that are thought to give rise to three-quarters of all cancer (Higginson and Muir, 1976). Besides the same potential problems with kinetics and interactions mentioned above, measuring genotoxicity in the field is complicated by the sheer numbers of agents that a human being or population come into contact with, especially over the decades it may require for cancer to appear. One approach has been to develop simple functional assays or “biosensors” that react to a spectrum of effectors rather than a single specific agent, such as a particular chemical. These instruments often use biological detectors, whole organisms or molecules such as antibodies or enzymes activated by interaction (binding) to xenobiotic agents to indicate the presence of such agents in the environment (Schubnell et al., 1999). This approach is still restricted by our understanding of the underlying mechanism of action of such agents, and again, the application to cancer usually involves the assumption of a genotoxic mechanism, although methods to detect possible agents acting through an epigenetic hormonal mechanism have also been developed (Seifert et al., 1998).

Biomarkers of Exposure: Internal Dose

To manifest a carcinogenic effect, most agents must be internalized within an organism and within a cell. “Biomonitoring” of potentially toxic exposures involves measurement of the agent in a tissue or bodily fluid readily available for sampling (Zielhuis, 1978). In experimental systems, a potentially toxic substance can be labeled and administered to the whole animal by various methods, and the uptake, distribution, persistence and elimination investigated by recovery of the label in urine and feces. In potential human exposures similar measures can be used to infer the magnitude and importance of the original dose. Such studies are complicated by the metabolism the original agent undergoes *in vivo*. Indeed, if the number and types of exposures humans normally undergo are daunting enough, the expansion of these effects through metabolism magnify the problem many fold. In an effort to mobilize and detoxify potentially toxic substances the body metabolizes or “biotransforms” them into more water soluble derivatives; unfortunately this often makes them more reactive and therefore more genotoxic, in effect also “activating” them. Thus, it is usually not only the original agent that must be monitored in bodily fluids, but a complex mixture of metabolites, which themselves have

different potentials for toxicity. This metabolism of chemical agents has become an important element in the individual "exposure modification" that must translate an exposure into a disease, and differences in the ability to metabolize chemicals have been shown to significantly affect their ultimate biological activity (Davies, 1988). Indeed, most molecular epidemiological studies of "genetic susceptibility" to genotoxic agents have involved functional or genetic markers of metabolic enzymes (Autrup, 2000; Guengerich, 2000). Considering the number of potential phase I (esterases, cytochrome P-450 monooxygenases, epoxide hydratase, etc.) and phase II (methyltransferases, sulfotransferases, acetyl transferases, glucuronyl transferases, glutathione-S-transferases, etc.) enzymes involved in this process it is difficult to predict the fate of a chemical in a biological system, although computational models have been developed (Ioannides et al., 1994; Klopman et al., 1994). There is an unfortunate tendency to look for associations between polymorphisms in these genes and health effects without ever determining whether the polymorphism has any effect on functionality. Since epidemiology by definition can only be hypothesis generating, demonstration of such an association should only provide further impetus for a functional analysis of the polymorphism and its mechanistic role in the disease process (DuPont et al., 1995; Traver et al., 1997).

Biomarkers of Exposure: Biologically Effective Dose

Genetic toxicology is a rather unique subspeciality of toxicology in that the target molecule, DNA, is neither cell-type- or organ-specific. Thus, a genotoxic effect, potentially contributing to carcinogenesis, can occur in almost any cell in the body. Certain non-genotoxic carcinogenic agents, such as transforming viruses and xenoestrogens are likely to be more restricted in the types of cells they can affect. Traditionally, genotoxicants have been defined rather narrowly as agents that interact directly with the DNA, despite the fact that agents affecting chromatin proteins, microtubules, etc. can affect DNA replication and chromosome segregation. Therefore measurement of the "effective" dose of a carcinogen has often been done by quantitating DNA adducts (or blood protein adducts as a surrogate). There are many methods to do this in bulk, but the most widely applied is ^{32}P -postlabeling, which yields "spots" of bases with altered migration in a two-dimensional chromatography system (Randerath et al., 1981; IARC, 1993, 1994). An advantage of this and similar detection systems is that they display all of the base adduction

products in a quantitative manner, such that all potential DNA damage can be estimated. The major disadvantage of such systems is that there are usually multiple species of adducted bases and, without individually characterizing each species, it is impossible to assign a relative importance to each spot. Although they must have a minimal persistence to be detectable at all (i.e. not removed from the DNA too quickly by DNA repair mechanisms), different altered bases can have very different effects on DNA replication and hydrogen bonding, and therefore on the types and amounts of resulting mutations. Recent studies have often targeted a single, well characterized adduction species with monoclonal antibodies; however, such studies assume that the total genotoxic effect of a mixed exposure can be estimated from a single mutagenic product, which is not likely to be consistent (Santella, 1999; Poirier and Santella, 2000).

Biomarkers of Disease: Generalized Biological Effect

In keeping with the genotoxicity paradigm for carcinogenesis, interaction of a toxic agent with DNA does not produce a long-term effect unless it results in an unreparable mutation, defined as any heritable change in the amount or structure of the genetic material. Since we are referring to genetic changes in somatic cells, “heritable” suggests viable clonal propagation of the mutation through subsequent mitotic “generations”. A large number of methods for detecting and quantitating somatic mutation have been proposed and, to some degree validated in retrospective studies (MacGregor et al., 1995). Some markers, such as micronuclei or dicentric chromosomes, are inherently inviable; they therefore serve as indicators of similar processes that leave the cell mutated but alive (sort of a biomarker of a biomarker). Other monitored events, such as sister chromatid exchange, result in no genetic damage or biological effects, but are thought to respond to agents that can, in addition, induce chromosome breakage and/or rearrangement. The best validated biomarker of somatic mutation is the cytogenetic detection of stable chromosome aberrations, which has been shown to be predictive of subsequent cancer in two independent prospective studies (Hagmar et al., 1994, 1998; Bonassi et al., 2000). These studies provide strong evidence that, while other processes may contribute to human carcinogenesis, induction of somatic mutation is an important factor in overall cancer incidence. Measurement of gene-specific mutation has also shown promise as an intermediate biomarker of biological effect.

Somatic Mutational Analysis

There are two well-established methods for measuring gene-specific *in vivo* somatic mutation in humans. Both involve mutation at a non-oncogenic surrogate locus chosen to allow detection of mutation with single hit kinetics. These well-characterized “reporter” genes are the X-linked gene coding for hypoxanthine-guanosine phosphoribosyl transferase (HPRT), a ubiquitously expressed purine scavenger enzyme, and the autosomal gene for erythrocyte glycophorin A (GPA), the most common sialoglycoprotein on the red cell surface, and the genetic determinant of the MN blood group. The HPRT gene has been used for many years as a selectable marker in mammalian cell culture (Chu and Malling, 1968), and this assay system has been adapted to T-lymphocytes in short term cultures derived from human peripheral blood (Strauss and Albertini, 1979; Morley et al., 1983). The GPA assay is designed to detect a wide range of potentially inactivating mutations at the GPA locus by flow cytometric analysis of peripheral blood erythrocytes (Grant et al., 1991; Grant and Bigbee, 1993). The two assays have complementary features (**Table 1**). The GPA assay is fast and inexpensive, utilizing flow technology to quickly quantify rare mutational events. The HPRT assay requires cell culture and drug selection, making it more expensive and labor-intensive. However, the GPA assay can only be performed in genetically informative MN heterozygotes, and the mutational basis of the phenotypic variation cannot be confirmed at the molecular level, whereas the HPRT assay can be performed in virtually anyone, in a multitude of cell-types, and can be used to generate “mutational spectra” that potentially can identify the inducing genotoxic agent. In previous studies utilizing both assays, the correlation between these biomarkers is consistently better than the correlation of either with physical or environmental estimates of exposure, presumably because both of these assays take into account both the extent of exposure and individual variation in response to genotoxic exposure (Grant and Jensen, 1993).

The GPA and HPRT assays have been extensively validated as quantitative measures of genotoxic exposures. Investigations include exposures to ionizing radiation, such as the survivors of the bombing of Hiroshima (Langlois et al., 1987, 1993; Hakoda et al., 1988), accidents such as Chernobyl (Jensen et al., 1995; Livingston et al., 1997) and Goiânia (Straume et al., 1991) and other medical (Nicklas et al., 1990; Grant and Bigbee, 1994), environmental

(Bridges et al., 1991) and occupational studies (Messing et al., 1989; Straume et al., 1992). Similarly, the response of these systems to chemical exposures, such as PAHs, and cigarette smoke has been established in a series of studies of environmental (Jones et al., 1993; Ammenheuser et al., 1994) and occupational exposures (Major et al., 1992; Compton-Quintana et al., 1993; Perera et al., 1993; Dubeau et al., 1994; Rothman et al., 1995). Given that these assays are sensitive to a wide range of genotoxins, it has been suggested that these measures of somatic mutation might provide a biomarker of cancer risk associated with genotoxic exposure (Albertini et al., 1993; Akiyama et al., 1995).

There have been three studies specifically designed to determine whether newly diagnosed cancer patients have higher somatic mutation frequencies than disease-free individuals, i.e. whether cancer incidence is associated with increased levels of gene-specific (as opposed to chromosomal) mutation. In 1989, a study of lung cancer patients with the HPRT assay demonstrated significantly higher mutant frequencies in the patient population versus controls (Tompa and Sapi, 1989). A subsequent study of breast cancer patients revealed HPRT mutant frequencies higher than controls and women with benign breast masses, but the differences failed to reach statistical significance (Branda et al., 1992). More recently, a significant increase in mutation at the GPA locus has been reported for a population of hepatocellular carcinoma patients (Okada et al., 1997).

Several other mutational studies of cancer patients have been performed using the GPA assay, usually to demonstrate the genotoxicity of the therapeutic regimen (Umeki et al., 1991; Hirota et al., 1994; Mott et al., 1994; Boyse et al., 1996). Our studies of this type have always involved analysis of both concurrent disease-free controls and a pre-therapy sample from each patient. When the results from these two populations are pooled and compared, the patients are significantly higher for total variant frequency (combining both allele-loss and loss-and-duplication classes) ($p < 0.01$) (**Figure 2**). These data include subpopulations of patients with breast (Bigbee et al., 1990), prostate (Grant and Bigbee, 1994) and testicular cancer (Perera et al., 1992). The HPRT assay has also been used extensively to demonstrate a genotoxic effect of cancer chemotherapy upon circulating lymphocytes. In addition to the two mentioned above, seven studies have been published in which the frequency of lymphocytes with mutations at the

X-linked HPRT locus was determined in newly diagnosed cancer patients prior to genotoxic therapy (Albertini, 1980; Lange and Prantner, 1982; Dempsey et al., 1985; Messing and Bradley, 1985; Sala-Trepat et al., 1990, Ammenheuser et al., 1991, Caggana et al., 1991). In all nine studies, the overall frequency of somatic mutation at the HPRT locus was higher in the cancer patients than in concurrent controls. When these data were reviewed and pooled for re-analysis (Cole and Skopek, 1994), the ~2-fold elevation in somatic mutation frequency demonstrated by this pooled data from cancer patients (N=187) was highly significant ($p < 0.001$) (**Figure 3**).

These data suggest that human carcinogenesis is associated with increased *in vivo* somatic mutation, and, based on the validation studies detailed above, that these mutation assays can act as integrative biodosimeters for genotoxic exposures. It is significant that the association seems to hold not just in tumors with a well-accepted mutagenic etiology, such as lung cancer, but also in tumor types with viral (hepatocarcinoma) or hormonal (breast, testicular, prostate cancer) components in their progression. This observation is consistent with the concept of a multi-step mutational pathway of carcinogenicity where one or a few steps can be fulfilled by epigenetic factors, but numerous other steps are still dependent on mutagenesis. Since these assays can measure both transient and persistent DNA damage, in the stem cell and differentiating hematopoietic compartments respectively, they show great promise as biomarkers of chemopreventive measures against genotoxicity, such as antioxidants.

The association of cancer incidence with a modest elevation in somatic mutant frequencies suggests that cancer can be caused by "normal" or "background" levels of genotoxic exposure. Individual variation in susceptibility to genotoxic insults would therefore become an important factor in determining whether mutagenesis, and subsequently carcinogenesis, would result from a particular exposure. The HPRT and GPA assays have also been applied to individuals and populations suffering from "DNA repair deficiency" syndromes, which are characterized by very high cancer incidences. Thus, HPRT mutation has been found to be spontaneously elevated in homozygotes for the recessive "cancer-prone" disorders Bloom syndrome (Vijayalaxmi et al., 1983), Fanconi anemia (Vijayalaxmi et al., 1985; Sala-Trepat et al., 1993) and ataxia telangiectasia (Henderson et al., 1986; Cole and Arlett, 1994), all associated with deficiencies in resolving DNA double-strand breaks. The GPA assay has demonstrated 10- (ataxia

telangiectasia), 50- (Fanconi anemia) and 100-fold (Bloom syndrome) increases in the frequency of spontaneous somatic mutation in these patients (Bigbee et al., 1989; Langlois et al., 1989; Kyoizumi et al., 1989; Sala-Trepat et al., 1990; Mott et al., 1994; Grant et al., 1998; Grant and Auerbach, in preparation). HPRT mutant frequencies appear to be elevated in xeroderma pigmentosum patients, which are characterized by a deficiency in nucleotide excision repair (Tates et al., 1989; Cole et al., 1992), but there is no evidence for such an increase at the GPA locus (Langlois et al., 1990). Both assays have demonstrated subtle elevations in mutant frequency in the premature aging disease Werner syndrome (Fukuchi et al., 1990; Moser et al., 2000). Thus, from these studies, an alternative explanation for the elevated mutation frequencies observed in the sporadic cancer patient populations described above, is that instead of sustaining slightly higher than normal genotoxic exposures, these individuals are characterized by manifesting slightly higher than normal genetic susceptibilities to genotoxic injury. This suggestion is similar to the proposal of Hsu (1983), that normal populations should show interindividual variability in DNA repair capacities, and that those with the highest susceptibility to unavoidable genotoxic exposures (but still within the range of normal), would be at greatest risk of developing cancer.

Mutagen Sensitivity

Hsu's own approach to demonstrating this principle was based on another characteristic of the cancer-prone syndromes: their hypersensitivity to DNA-damaging agents (Auerbach et al., 1979; Paterson and Smith, 1979; Weksberg et al., 1979). This cellular phenotype has been exploited to map and clone the underlying genes responsible for these conditions, and lymphocyte mutagen hypersensitivity continues to be used as a definitive diagnostic laboratory test. Hsu conjectured that milder forms of this "mutagen sensitivity" phenotype should occur in the human population, and might contribute to the incidence of "common" tumors in the "normal" population. He adapted the mutagen sensitivity tests developed for diagnosis of the DNA repair deficiency diseases into a screening tool based on the induction of transient cytogenetically detectable chromatid breaks (Cherry and Hsu, 1983; Hsu et al., 1985, 1989, 1991). These studies demonstrated significant interindividual variation in response of the disease-free population to a known genotoxic agent, the radiomimetic DNA cross-linking agent bleomycin. They also

demonstrated that a significantly greater proportion of individuals manifesting a number of different types of cancer were "hypersensitive" to this mutagen, in that they suffered more DNA damage when their lymphocytes were exposed to a standard dose of bleomycin. This work has been carried forward by Spitz and co-workers in a series of studies demonstrating that bleomycin sensitivity is associated with risk of head and neck (Spitz et al., 1989, 1993; Cloos et al., 1996) and lung (Wu et al., 1995, 1998) cancer. Hsu et al. (1993a, b) introduced the idea that sensitivity to other mutagenic chemicals could also be measured by induction of chromatid breaks. In these studies the inducing agent was 4-nitroquinoline-1-oxide (4NQO), which causes the same type of DNA damage as UV light, the genotoxic agent implicated in skin carcinogenesis. This principle has subsequently been applied in the lung cancer study using the polyaromatic hydrocarbon and tobacco smoke mutagen benzo[a]pyrene diol epoxide (BPDE) as the inducing agent (Li et al., 1996; Wei et al., 1996).

Biomarkers of Disease: Specific Biological Effect

Just as some would argue that an adduct is not important unless it results in a mutation, there are those that would argue that the mutation is not important unless it is involved in the progression of the disease. Screening for mutations in oncogenes and segregation of tumor suppressor genes (Grant, 1992) blurs the distinctions of public health concerns, such as identifying individuals at increased risk of cancer, and purely medical concerns, i.e. the early detection of the disease itself. Whatever the intent of the study, it can take the form of a screen because of the early observation that tumor cells (and potentially preneoplastic cells as well) can be found in many fluids and excretia of the body (Papanicolaou, 1949). Advances in cytological techniques, and the development of antibodies to cell lineage markers and carcinoembryonic antigens maintained interest in these cells, but it wasn't until the delineation of the role of somatic mutation in oncogenesis that the possibility of molecular screening arose. Thus, there has been much interest and some progress in the last decade towards using the molecular detection of so-called "early mutations" in such biological samples as buccal swabs, mouth rinses, lung lavage, urine, feces, etc. as diagnostic and prognostic markers (Sidransky, 1997). More recently, it has been found that free circulating DNA in serum, long known to be at higher levels in cancer patients (Leon et al., 1977; Shapiro et al., 1983), is primarily derived from necrosing and apoptosing cells (Stroun

et al., 2000; Jahr et al., 2001). Both activated oncogenes (Sorenson et al., 1994; Anker et al., 1997) and segregation of tumor suppressor genes (Chen et al., 1996; Nawroz et al., 1996), reflective of genetic changes in the primary tumor, have been detected by analysis of DNA amplified from blood samples from cancer patients.

Conclusion

In many ways, the fields of molecular toxicology and molecular epidemiology are in a holding pattern. There has been a general reluctance to leave the so-called "validation" phase, where potential biomarkers are evaluated in populations with known (and usually extreme) exposures and predispositions and move these studies into the general population, and subsequently into clinical or public health practice. For basic scientists, this involves taking on responsibilities for interaction with human populations and individuals that some researchers may not appreciate. From the clinical side, in the absence of an established intervention, there may be little reason or even justification for "predicting" disease. Only through application of those biomarkers that do exist, such as the promising technologies discussed in this report, can the basic scientist become comfortable with such translational research, and can preventive measures be developed to provide the practitioner with an armamentarium to "treat" preneoplastic disease.

The biomarkers available and in development for cancer reflect, to a large degree, the inclinations of toxicologists to equate mutagenesis with carcinogenesis. Indeed, data presented here suggest that although other mechanisms are known to contribute to cancer, mutation, both chromosomal and gene-specific, appears to be involved in all cancers. This in turn suggests that if you live long enough, you will inevitably develop cancer, and that the specific type of cancer will be the one you are most susceptible to, due to the types of exposure you have sustained, as well as your underlying genotype. Thus, a certain level of genotoxic effect may be sufficient to cause hepatocarcinoma in an individual with a chronic hepatitis B infection, but a slightly later onset kidney tumor in an individual without such a viral predisposing factor. The strength of the mutational model of cancer lies in its ability to rationalize itself with these other factors. We know, as mentioned above, that viruses can deliver an activated oncogenes (retroviruses), or provide a protein sink for tumor suppressor gene products (animal viruses) (Butel, 2000).

Genetic factors in cancer have been found to provide congenitally a mutation that traverses one step in the carcinogenic pathway (Knudson, 1992), or confer a "mutator" phenotype causing a more rapid progression through the pathway (Bhattacharyya et al., 1994), or both. Hormonal factors, even if they are not overtly genotoxic, can mimic mutation via their effects on transcriptional regulation (Webb et al., 1999), or affect mutation rates as suggested by the "mitogen-mutagen" hypothesis (Henderson and Spenser Feigelson, 2000). Toxicologists must also be willing to expand their definition of a mutagen; for example, since aneuploidy is unquestionably a mutational event, agents that cause it through interaction with centromeric proteins or microtubules (as opposed to direct interaction with DNA) should be considered mutagens. Despite the present success of mutationally-based biomarkers, we must be aware that application of such surrogate end points for cancer depends on the confidence the entire field feels in the underlying mechanistic basis of cancer. Some clinicians maintain that the only credible intermediate biomarker for carcinogenesis, especially for prospective trials of chemoprevention, is the appearance of preneoplastic lesions (Einspar et al., 1997); this despite the fact that the vast majority of such lesions do not, and perhaps cannot develop into malignant tumors (Pretlow et al., 1995). Mutational biomarkers have also been criticized for not discriminating between exposure and susceptibility, or for not being more agent-specific. The best reply to such criticisms is to apply the markers we have now in the most appropriate way, and if such discrimination is found to be important, to continue to develop methods to further specify the relative contributions of each factor in each particular disease or lesion.

One final thought. All of the preceeding is based on the assumption that toxicology, pathology etc., will continue to play an important role in oncology. History, however, suggests that science, even medical science, tends to follow the fad of the latest technology, even when it is not necessarily appropriate. With the recent completion of the first phase of the human genome project, we have entered into a period of increased enthusiasm for genomic research that may or may not complement the types of research discussed in this report. We mentioned earlier that every cell from an 80 year old man still carries his entire genetic code, facilitating such genomic research, even in such late-onset diseases as cancer. Our 80 year old man also has a complete record of his lifetime accumulated exposures, at least genotoxic exposures, in his cells, although different aspects may be found in different cell types, locations, etc. We must develop methods

of rapidly screening an individual for evidence of cumulative past exposure that can be used to characterize their level of response. The justification most often given for the extensive involvement of the U.S. Department of Energy in the human genome project was, essentially, how can we identify mutations unless we know what the normal gene sequence is? We now need to take up this challenge and use the technologies developed for charting the evolution of the hereditary genome through generations to begin to map the changes in the somatic genome that occur over a normal lifetime, and during the carcinogenic process.

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Figure 1. Epidemiology of induced human disease in the mechanistic context of molecular toxicology. Insights into the absorption, distribution, metabolism and elimination of environmental agents are combined with insights into the mechanism of the disease process itself to provide potential intermediate steps in the progression that can be tested for validity and applied as surrogates for the eventual health effect (after Hulka and Wilcosky, 1988; Schulte, 1989; Hulka, 1991; Perera et al., 1993).

Figure 2. Comparison of *in vivo* somatic mutation at the GPA locus in a population of untreated patients with diverse types of cancer and disease-free controls.

Figure 3. Comparison of *in vivo* somatic mutation at the HPRT locus in a population of untreated patients with diverse types of cancer and disease-free controls.

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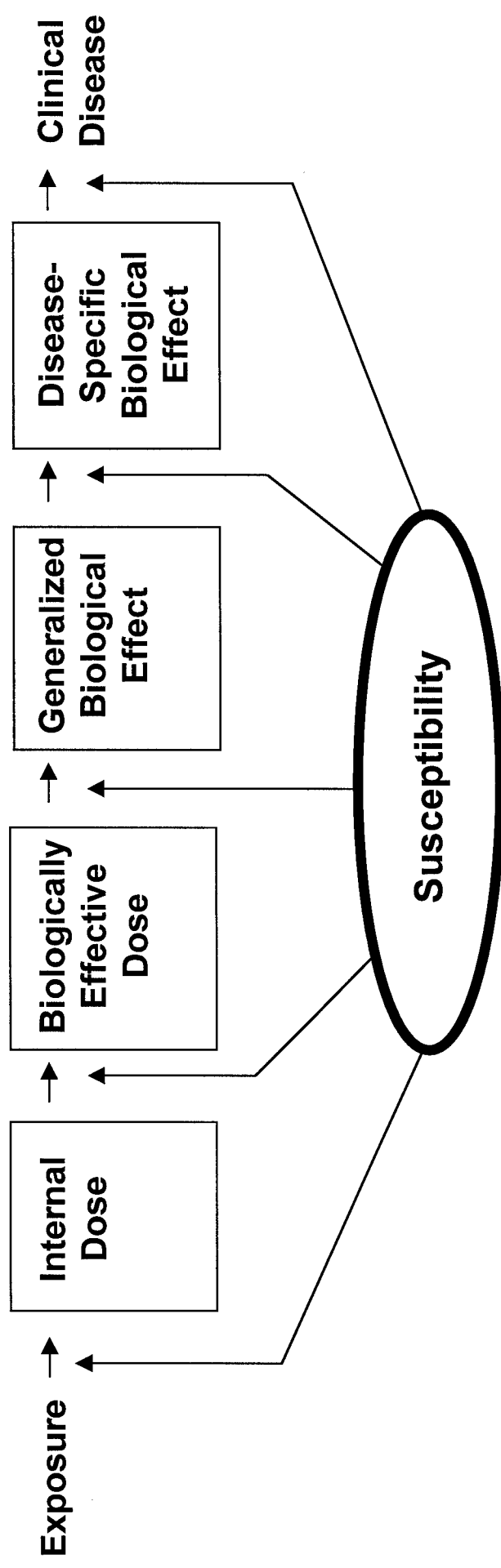


Figure 1

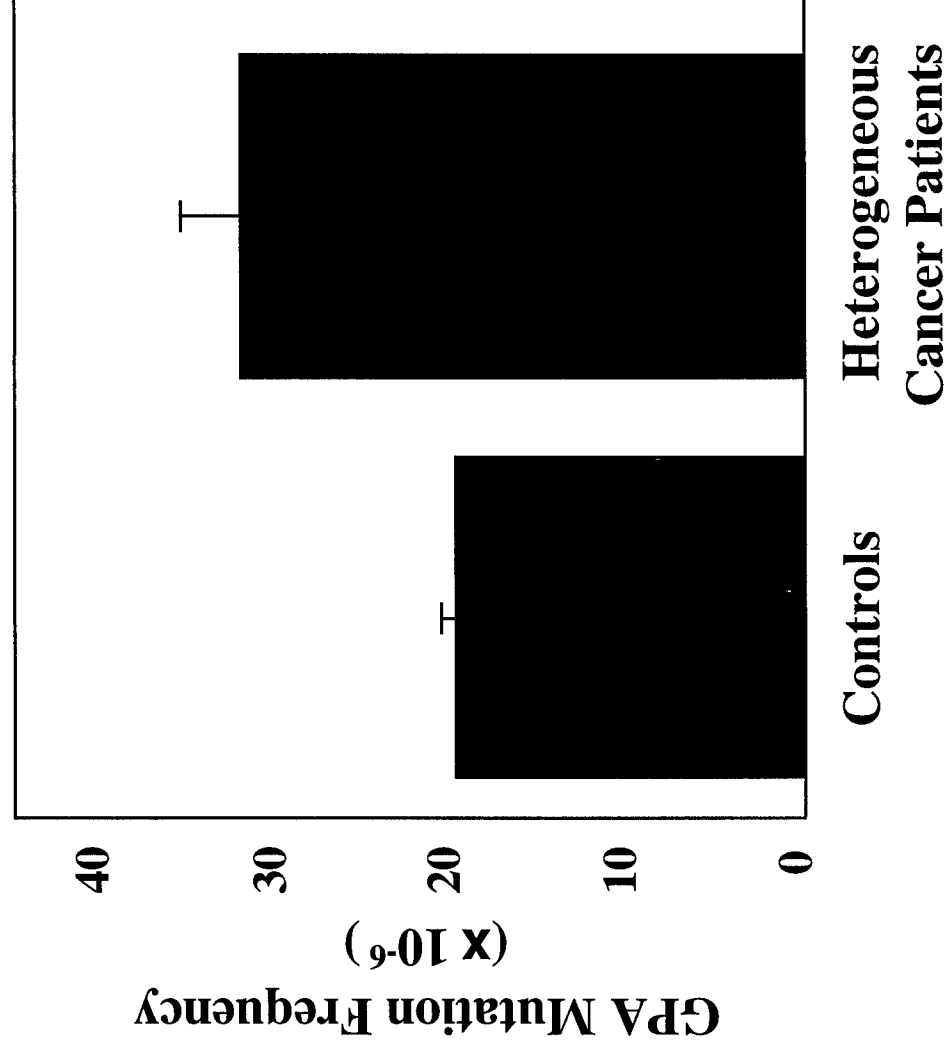


Figure 2

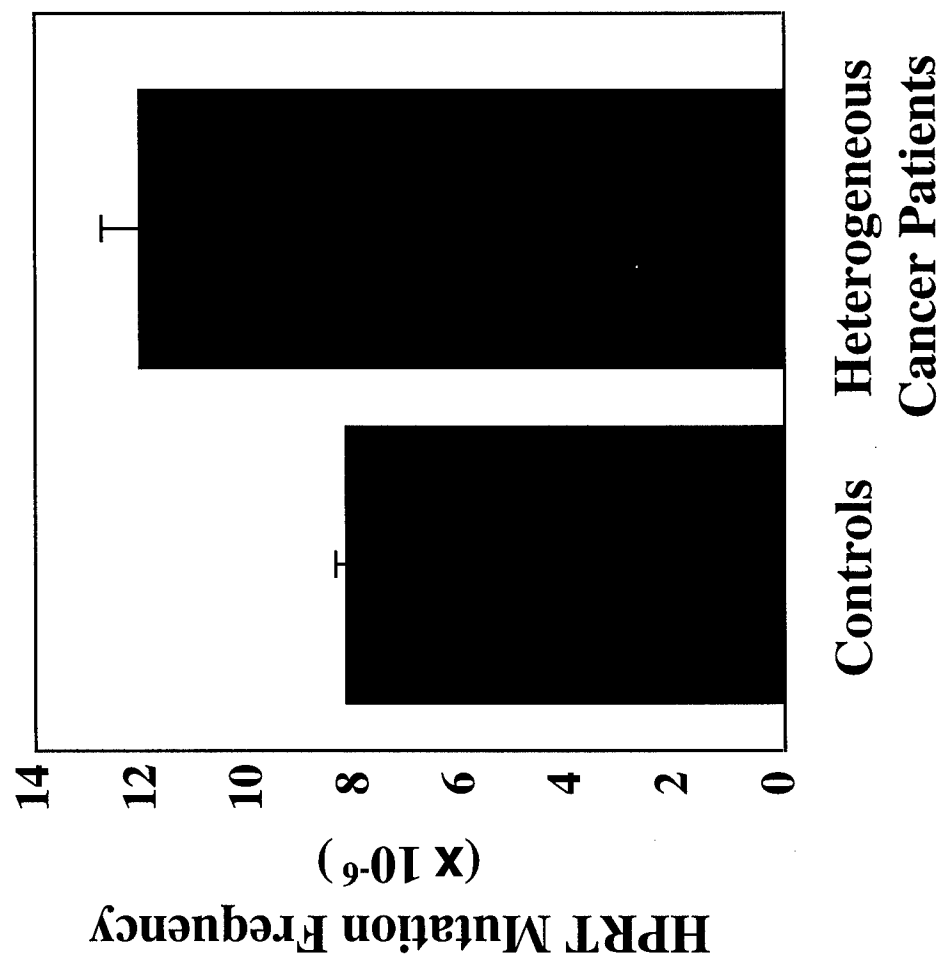


Figure 3